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Functional variants of sphingosine-1-phosphate receptor 1 gene associate with asthma susceptibility

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Abstract

Background—The genetic mechanisms underlying asthma remain unclear. Increased permeability of the microvasculature is a feature of asthma and the sphingosine-1-phosphate receptor, S1PR1, is an essential participant regulating lung vascular integrity and responses to lung inflammation.

Objective—We explored the contribution of polymorphisms in the S1PR1 gene (*S1PR1*) to asthma susceptibility.

Methods—A combination of gene re-sequencing for SNP discovery, case-control association, functional evaluation of associated SNPs, and protein immunochemistry studies was utilized.

Results—Immunohistochemistry studies demonstrated significantly decreased S1PR1 protein expression in pulmonary vessels in asthmatic lungs compared to non-asthmatic individuals ($p < 0.05$). Direct DNA sequencing of 27 multiethnic samples identified 39 *S1PR1* variants (18 novel SNPs). Association studies were performed based on genotyping results from cosmopolitan tagging SNPs in three case-control cohorts from Chicago and New York totaling 1061 subjects (502 cases and 559 controls). Promoter SNP rs2038366 (–1557G/T) was found to be associated with asthma ($p = 0.03$) in European Americans. In African Americans, an association was found for both asthma and severe asthma for intronic SNP rs3753194 (c.–164+170A/G) ($p = 0.006$ and $p = 0.040$, respectively) and for promoter SNP rs59317557 (–532C/G) with severe asthma

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($p=0.028$). Consistent with predicted *in silico* functionality, alleles of promoter SNPs rs2038366 (–1557G/T) and rs59317557 (–532C/G) influenced the activity of a luciferase *S1PR1* reporter vector in transfected endothelial cells exposed to growth factors (EGF, PDGF, VEGF) known to be increased in asthmatic airways.

Conclusion—These data provide strong support for a role for *S1PR1* gene variants in asthma susceptibility and severity.

Clinical Implications—Our results indicate *S1PR1* is a novel asthma candidate gene and an attractive target for future therapeutic strategies.

Capsule summary—This study identified novel polymorphisms in *S1PR1*, revealed the functional implications of *S1PR1* genetic variants in different populations, and their association with asthma susceptibility and severity.

Keywords

asthma; sphingosine-1-phosphate receptor 1; single nucleotide polymorphism; promoter activity

INTRODUCTION

Asthma is a chronic inflammatory lung disease, characterized by airway inflammation, increased vascular permeability¹ and subsequent airway remodeling^{2–4}. Recently, the influence of angiogenic factors such as vascular endothelial growth factor (VEGF)⁵ on asthma pathobiology has emerged with increased levels of VEGF observed in asthmatic airways⁶ likely resulting in increased microvascular permeability, mucosal edema and airway diameter narrowing, thereby amplifying the effect of airway smooth muscle contraction⁷. Platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) levels are also increased in asthmatic airways^{8,9} mediating airway smooth muscle proliferation and asthma remodeling^{10,11}.

We previously demonstrated that another angiogenic factor^{12,13}, sphingosine 1-phosphate (S1P), a multifunctional lipid mediator, is essential for regulation of vascular integrity and restoring vascular barrier function under inflammatory states¹⁴. S1P rapidly stimulates vascular endothelial cells and smooth muscle cells via ligation of a G protein-coupled S1P receptor family including sphingosine 1-phosphate receptors 1 to 5. Stimulation of the sphingosine-1-phosphate receptor 1 (*S1PR1*), which is highly expressed in vascular endothelial cells^{15,16}, results in barrier stabilization or reversal of barrier disruption induced by edemagenic agents, events driven by robust Rac GTPase-mediated cytoskeletal rearrangement¹⁴. In murine and canine models, ligation of *S1PR1* also attenuates lung vascular permeability induced by LPS, ischemia/reperfusion, excessive mechanical ventilation and radiation^{17–19}. Disruption of the *S1PR1* gene (*S1PR1*^{–/–}) in mice resulted in embryonic lethality due to massive vascular permeability and hemorrhage at –12.5 to –14.5 embryonic day^{20,21}. Furthermore, heterozygous *S1PR1*^{+/-} or wild type mice pretreated with a *S1PR1* antagonist reduce S1P-mediated barrier protection following LPS challenge^{17,18,22}. Mice engineered to selectively lack S1P in plasma displayed increased vascular leak which was associated with increased inter-endothelial cell gaps in venules, and the addition of an agonist for the *S1PR1* rescued the vascular integrity²³.

Direct roles for S1P and S1P receptors such as *S1PR1* in asthma are inconclusive. Elevated levels of S1P have been identified in bronchoalveolar lavage (BAL) fluid from allergic asthma patients after ragweed antigen challenge, suggesting that S1P may serve a potential proinflammatory function in asthmatic airways²⁴ and participate in airway hyperresponsiveness via modulation of airway smooth muscle cell function and autocrine

mediator of activated mast cells^{25–27}. In contrast to these potentially detrimental effects of S1P, intratracheal administration of FTY720, a fungal derivative and S1P analogue, significantly suppresses Th2-dependent eosinophilic airway inflammation and bronchial hyperresponsiveness, the cardinal features of asthma²⁸. Modulation of S1PR1 expression also regulates immune-cell trafficking through transactivation; through internalization, ubiquitination and subsequent degradation; or through interaction with the lymphoid cell surfaces molecule CD69²⁹.

Given the complex mechanisms involved in *S1P/S1PR1* participation in asthma and the emerging importance of barrier regulation in asthma pathobiology, we hypothesized that *S1PR1* genetic variants may modify asthma susceptibility and severity. Our results indicate *S1PR1* as a novel asthma candidate gene and an attractive target for future therapeutic strategies.

METHODS

Immunohistochemistry study

Postmortem formalin-fixed paraffin-embedded lung tissue sections (5µm) were obtained from seven asthmatics (3 European descent Americans (EAs) and 4 African descent Americans (AAs)) and seven non-asthmatics (3 EAs and 4 AAs) involved in fatal motor vehicle accidents. S1PR1 protein expression was detected using rabbit polyclonal antibodies against human S1PR1 (sc-25489, Santa Cruz, CA) in 1:150 dilution by an indirect immunoperoxidase method with Envision + Kits (DACO, Carpinteria, CA) and counter-stained by H&E. All slides were coded and scanned in blinded fashion. A minimum of five areas in the subepithelial region were analyzed and the total S1PR1 content determined per unit area (104 µm²) using a computerized image analysis system (ImageJ, NIH)^{30, 31}. The quantity of endothelial S1PR1 expression was normalized by endothelial marker VE-cadherin detected with mouse monoclonal antibodies in 1:50 dilution (sc-9989, Santa Cruz, CA).

Human S1PR1 gene re-sequencing

DNA samples of 27 unrelated non-asthmatic individuals (14 AAs and 13 EAs) were sequenced to search for common variation in the *S1PR1* gene. DNA sequencing protocols and polymorphism identification were performed by Polymorphic DNA Technologies (Alameda, CA). PCR primers were designed to amplify the entire gene and 2 kb of the predicted promoter (Genomatix Software Inc, Ann Arbor, MI) and downstream regions of the gene. A list of the primer pairs utilized is provided in Supplement Table S1. The genomic sequence NM_001400 was used as the reference sequence and variation reported following established guidelines³².

Study populations and demographics

A total of 110 EAs and 192 AAs unrelated asthma cases and 209 EAs and 193 AAs controls were collected in Chicago as part of the Collaborative Study on the Genetics of Asthma protocol³³. Chicago controls were adults reporting a negative personal and first-degree family history for asthma reporting either at least three grandparents of African American ancestry or of European ancestry. They should be considered population-based controls since medical histories were not obtained, and medical testing was not performed. A detailed clinical description of case samples has been reported previously^{34, 35}. Additionally, samples from 200 asthma cases and 157 controls from central or west Harlem, New York (all self-reported as African Americans), were enrolled as part of population-based asthma epidemiology studies at Harlem Hospital Center for asthma exacerbations³⁶. In New York samples, affection status, both in cases and controls, was determined by qualified physicians

using standard diagnostic criteria³⁷. Severe asthma was determined using the National Asthma Education and Prevention Program Guidelines (1997), based on night time symptoms, asthma exacerbations and a forced expiratory volume (FEV₁) < 60% predicted at any time. Protocols were approved by the Institutional Review Board and written informed consent was obtained from all participants.

Genotyping

Genotyping was conducted using the iPLEX Gold™ Platform following manufacturer's protocol (Sequenom, San Diego, CA) except for SNP rs1411017, rs61780687, and rs3737577, which were genotyped using TaqMan™ allelic discrimination assays (Applied Biosystems, Foster City, CA). Briefly, iPLEX™ assays were scanned by MALDI-TOF mass spectrometry and individual SNP genotype calls were automatically generated using Sequenom TYPER 3.4™ software. TaqMan genotyping was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems) with automated calls generated using the SDS software based on discriminating plots (95% confidence). Approximately 10% of the samples were genotyped by duplicate to monitor genotyping quality. Genotyping was blind to case-control status and the ethnic background of the sample.

Assessment of genomic ancestry

Samples collected in Chicago had been previously assessed for 30 highly informative microsatellites of individual ancestry to European, West African and Native American/Asian populations^{38, 39} selected from about 2,000 microsatellites genotyped in a previously described multiethnic cohort of 35 European Americans, 88 African Americans, 34 Chinese and 29 Mexican Americans⁴⁰.

To estimate individual ancestries of New York participants, all samples were genotyped for 96 ancestry informative markers (AIMs) selected for being highly divergent ($\delta > 0.6$) among European, West African and Native American/Asian populations^{41, 42}. Supplement Table S2 shows a list of the AIMs used and frequency divergences among reference populations used to derive the estimates of this study (60 Europeans and 131 West Africans). Kosoy *et al*⁴³ showed that subsets as small as 24 AIMs of this panel were useful for ascertaining the origin of subjects from different continents among American populations.

Individual ancestries were estimated from multilocus genotype data using the Bayesian Markov Chain-Monte Carlo (MCMC) method implemented in the program STRUCTURE 2.1⁴⁴. STRUCTURE 2.1 was run under the admixture model using prior population information and independent allele frequencies. The MCMC method using a predefined number of parental populations ranging from K=1 to K=3 and a burn-in length of 50,000 for 70,000 repetitions was utilized. Note that while both AIM sets used are discriminatory for European, West African and Asian/Native American ancestry, the program was used to determine the number of sub-populations, K=2 or K=3, in the African American samples. The results showed that the model with the best likelihood was K=2 sub-populations showing a significant West African and European ancestral proportions in our case-control samples, congruent with previous observations^{38, 45, 46}. Summary statistics for the estimated European ancestry for all population groups is supplied in Supplement Table S3.

Statistical analysis

The multiple-marker selection algorithm, haplotype r^2 , included in TagIT 3.03 software⁴⁷ was used to select a set of cosmopolitan tagging SNPs (tSNPs) under previously described criteria³⁸. This algorithm maintains the tSNPs prediction accuracy and increase tagging efficiency by selecting fewer tSNPs than other methods⁴⁸. Departures from Hardy-Weinberg equilibrium (HWE) were tested by means an efficient exact test⁴⁹. All individual SNP

associations with disease were assessed using the Cochran-Armitage trend test with pre-specified scores of 0, 1 and 2 as risk probabilities based on the copies of risk alleles in the genotypes (i.e. assuming an additive model), and the empirical p -value obtained by 1000 permutations. All comparisons were performed against controls. Both HWE and trend test statistics were computed by means of a custom script for STATISTICA (StatSoft Inc., Tulsa, OK). In order to judge the significant SNP associations in the context of the multiple comparisons performed, a False Discovery Rate was assessed by QVALUE⁵⁰, not considering the two conditions tested due to the non-independent nature of both traits. SPSS 14.0 (SPSS Inc., Chicago, IL) was used for multiple logistic regression analysis and to obtain the odds ratio (OR) and its 95% confidence interval (CI) as measures of the strength of the effects. For Chicago samples, individual ancestry was the only covariate considered to adjust in logistic regression models since no further data was shared among controls and cases, although extensive phenotype characterization was performed for the latter³³. For New York samples, age, gender and smoking status along with the estimates of ancestry were used for the adjustments in logistic regression models. The Student t -test was used for comparison of promoter activity and protein expression between different groups. Statistical significance was set at $p < 0.05$.

Subcloning, transfection and luciferase activity assay

DNA fragments of the *S1PR1* gene containing the alleles rs2038366_G (−1557G) or the rs59317557_C (−532C) were amplified by PCR using the human genomic DNA and modified subsequently by site-direct mutagenesis to generate rs2038366_T (−1557T) and rs59317557_G alleles (−532G), respectively. All amplicons were verified by direct sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and fused to a pGL3-basic reporter vector (Promega, Madison, WI) containing a genetically engineered firefly luciferase gene without a promoter. Human pulmonary artery endothelial cells (HPAEC) (Cambrex, Walkersville, MD) were cultured in EBM-2 complete medium at 37°C, 5% CO₂, 95% air, within passages 6–10 as described previously^{14, 51}. Plasmid constructs were transfected into HPAEC with FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN) in Opti-MEM medium for 2 hrs. A plasmid with renilla luciferase gene (phRL-TK) was co-transfected with pGL3 fused constructs as transfection efficacy control. Cells were harvested 48 hrs post-transfection and lysed in Passive Lysis Buffer. Luciferase activity was measured by Dual-Luciferase Assay Kits (Promega). The relative promoter activities were expressed as the ratio of firefly luciferase in pGL3 to renilla luciferase in phRL-TK (RLU) following the Dual-Glo luciferase reporter assay protocols recommended by the manufacturer. Five independent transfections and duplicate luciferase assays were performed for each condition, along with pGL3-basic vector used as a control. The effects of specific growth factors (PDGF 50 ng/ml, VEGF 50 ng/ml, EGF 100 ng/ml) on luciferase activity assays were performed to assess the regulation of transcription factors (Egr2 or EGFR) related to *S1PR1* promoter SNPs.

RESULTS

S1PR1 protein levels are decreased in the lung vasculature of asthmatics

Initial studies examined the tissue protein expression of S1PR1 in normal and asthmatic lungs. Immunohistochemistry studies detected S1PR1 protein expression both in alveolar epithelium and lung vascular endothelium (Figure 1A). In pulmonary arteries from asthmatic subjects, S1PR1 protein expression was significantly decreased when compared to non-asthmatics ($p < 0.05$) (Figure 1B). The levels of S1PR1 protein were not significantly different between AAs and EAs samples within the small disease groups (data not shown).

Identification of novel S1PR1 polymorphisms

Re-sequencing of the entire *S1PR1* gene (except for a 307 bp repetitive stretch of the entire ~1700 bp intronic region) and 2kb of the upstream and downstream regulatory regions (a total of 8774 bp per sample) identified 39 variant sites (35 single base changes and 4 indels) with 18 variants not previously identified (Table 1). Among the novel variants, two resided in the proximal promoter region (–616C/T, and –485G/C), and one variant predicted a non-synonymous amino acid change in the coding region (Ala11Asp). A total of 10 variants (thereafter tSNPs) (Table 1) were sufficient to provide an adequate coverage of common *S1PR1* gene variation in both EAs and AAs and were used to genotype case-control samples described in the Materials and Methods section.

Polymorphisms of S1PR1 are associated with asthma in three case-control samples

Table 2 shows the minor allele frequency for the selected tagging *S1PR1* gene SNPs (tSNPs) by population group. Duplicate genotypes did not demonstrate discrepancies with the original genotypes (0% discordance rate). Details of HWE results can be found in Supplement Table S4. We noted that SNP 29 showed strong departures from HWE in both EAs and AAs from Chicago and was monomorphic in New York AAs. Thus, this SNP was not considered for further analyses. SNP rs17123892 was monomorphic in EAs. Samples with genotyping completion rate <80% were also removed. Final analyses were conducted for 8 tSNPs (exclusion of SNP29 and rs17123892) in 104 EA cases (27 with severe asthma) and 197 EA controls, and 9 tSNPs (exclusion of SNP29) in 184 AA cases (103 with severe asthma) and 191 AA controls from Chicago, and 195 AA cases (20 with severe asthma) and 139 AA controls from New York (Figure 2).

In EAs from Chicago, the promoter SNP rs2038366 (–1557G/T) was associated with asthma susceptibility ($p=0.013$, $q\text{-value}=0.10$) (Figure 2a). The association remained significant after adjusting for ancestry in a logistic regression model (adjusted risk per allele T was $OR=1.48$, $95\%CI=1.03\text{--}2.14$, $p=0.036$). None of the tSNPs resulted in significant association with severe asthma in EAs. Asthma susceptibility in AAs from Chicago was associated with the intronic SNP rs3753194 (c.–164+170A/G) ($p=0.005$, $q\text{-value}=0.045$) (Figure 2b) and remained significant after adjusting for ancestry in a logistic regression model (adjusted risk per allele G was $OR=3.00$, $95\%CI=1.37\text{--}6.60$, $p=0.006$). Moreover, this SNP was also significantly associated with severe asthma ($p=0.027$, $q\text{-value}=0.24$) (Figure 2b) after adjusting for ancestry ($OR=3.03$, $95\%CI=1.12\text{--}8.18$, $p=0.028$). In AAs from New York, the intronic SNP rs61780687 (c.–163-730A/T) showed significant association with asthma susceptibility ($p=0.043$, $q\text{-value}=0.39$) (Figure 2c) although it was not significant after adjusting for ancestry ($OR=1.58$, $95\%CI=0.88\text{--}2.85$, $p=0.124$). However, a promoter SNP rs59317557 (–532C/G) was identified to be significantly associated with severe asthma ($p=0.027$, $q\text{-value}=0.24$) (Figure 2c) and association remained significant after adjusting for ancestry (adjusted risk per allele C was $OR=2.53$, $95\%CI=1.10\text{--}5.80$, $p=0.028$) and for all available covariates ($OR=2.72$, $95\%CI=1.00\text{--}7.41$, $p=0.050$).

S1PR1 Promoter SNPs –1557G/T and –532C/G reside within predicted binding sites for transcription factors and potentially alter growth factor induced S1PR1 promoter activity

In order to explore the functional regulatory mechanism(s) of the associated *S1PR1* promoter SNPs, we performed transcription factor motif analysis (TRANSFAC) to search for transcriptional factor binding site(s) which could be potentially interrupted by these variants. *In silico* analysis revealed that epidermal growth factor receptor (EGFR) binding sites, encompassing the flanking sequences –1562 to –1552, are potentially interrupted by SNP - 1557T possibly resulting in loss of EGFR binding efficiency as compared with the common allele –1557G. Similarly, the –532G allele potentially interrupts early growth

response gene 2 (Egr2) binding to *S1PR1* promoter compared to the –532C allele (Figure 3A). We next utilized a luciferase activity assay to assess the allelic effects of SNPs –1557G/T and –532C/G on *S1PR1* promoter activity. Under the basal conditions (no stimulation), the activity of the *S1PR1* promoter with –1557T was significantly decreased compared to –1557G ($p<0.05$) (Figure 3B, **left panel**). However, luciferase activity of –532G was significantly increased compared to –532C ($p<0.05$) under basal conditions (Figure 3C, **left panel**). These data confirmed that variation of promoter SNPs at –1557T and –532G altered *S1PR1* gene expression. Next we tested if variation at –1557 affected *S1PR1* gene transcription in response to epidermal growth factor stimulation. Luciferase activity in endothelial cells transfected with either –1557G or T constructs followed by exposure of 100ng/ml EGF for 2 hours resulted in significant increased reporter activity compared to without EGF stimulation ($p<0.05$). However, activity of the promoter containing –1557T exhibited significant attenuated response to EGF compared to –1557G ($p<0.05$) (Figure 3B, **right panel**).

Finally, we assessed another variation at –532 affected *S1PR1* gene transcription in response to growth factor activation of the binding Egr2 site. Growth factors PDGF or VEGF (50ng/ml 2 hours) significant increased luciferase activity driven by both –532C and –532G compared to activity without growth factor stimulation ($p<0.05$). However, growth factor-mediated increases were significantly more pronounced for the *S1PR1* promoter containing –532G allele as compared to –532C allele ($p<0.01$) (Figure 3C).

DISCUSSION

We examined the role of sphingosine 1-phosphate receptor 1 (*S1PR1*) in asthma leveraging prior studies which revealed its ligand, sphingosine 1-phosphate (S1P), a multifunctional lipid mediator and an angiogenic factor to regulate key pathophysiologic features of asthma including airway hyper-responsiveness, airway remodeling and vascular permeability. *S1PR1* is a dominant S1P receptor in many tissues and an important regulator that in addition to its role in lymphocyte trafficking, regulates vascular permeability by endothelial integrity¹⁵, regeneration^{12, 21}, vasodilation⁵² and migration⁵³ etc. Our immunohistochemistry studies demonstrated that *S1PR1* protein expression in pulmonary arteries significantly decreased in asthmatic lungs, suggesting that asthma developing is significantly associated with decreased *S1PR1* in endothelium. Subsequently, we explored the functional contribution of *S1PR1* gene (*S1PR1*) polymorphisms to asthma susceptibility and severity by a combination of *S1PR1* gene re-sequencing for SNP discovery, case-control association studies, and luciferase assays. We identified *S1PR1* variants to be associated with asthma or severe asthma across unique case-control samples.

A strength of this study is that we performed an initial description of the common gene variation in a multiethnic panel of samples before selecting a cosmopolitan set of tagging SNPs to allow adequate coverage of other common untyped variants for the association analysis. More importantly, an additional strength is that gene association was found across three independent case-control samples totaling 1061 subjects (502 cases and 559 controls). Moreover, we formally assessed the risk of false SNP associations in the context of the multiple comparisons and tested whether the association results might be affected by population stratification. The latter was performed by genotyping highly informative ancestry informative markers and estimating proportions of European and West African descent to be used in logistic regression modeling. Congruently, two of the associated SNPs demonstrated an effect in *in vitro* endothelial cell luciferase assays, providing strong functional evidence for the significant role of *S1PR1* gene in asthma. It is worth noting that although associated SNPs are not in linkage equilibrium, these SNPs show poor correlation across European and African American samples (maximum $r^2=0.22$). Thus, in addition to

the strong evidence involving S1PR1 in asthma, our data provide support that *S1PR1* gene variants associate with asthma susceptibility. Genetic and/or environmental heterogeneity, variability in phenotype definitions, interactions of an untested causal variant with the associated SNPs⁵⁴, small effects sizes or inappropriate power could explain the failure to replicate findings at individual SNPs. In support of this latter possibility, although none of the individual SNP associations achieved statistical significance in all case-control samples, the direction of the effect for asthma susceptibility was consistent for one of the functional SNPs, rs2038366 (–1557G/T), across all three samples studied (OR_{GT}: 1.11 [95%CI: 0.84–1.49]; OR_{TT}: 1.68 [95%CI: 1.08–2.61], Mantel-Haenszel test $p=0.045$).

Our study has several limitations which need to be recognized including that Chicago controls, not clinically characterized for asthma, may lead to a reduction in power. However, it is well known that the consequences of misclassification of cases as controls are modest for a trait such as asthma (power reduction average <4%)^{55, 56}. We used a tagging approach after deep gene re-sequencing to study the association of *S1PR1* gene with asthma. However, it is unclear whether the associations observed are due to a few or all of these SNPs, or to other nearby variants within or flanking the gene. We designed this study to explore the association of common variants in the region and not to analyze the effect of rare variants, for which the study is underpowered. Thus, considering that re-sequencing of a few dozen samples is not sufficient for revealing all variation of the gene, it is likely that other variation within or outside the explored region may also contribute to disease susceptibility as no single SNP contributed to asthma in all populations. Conversely, such heterogeneity has been previously shown for a polymorphism firmly associated with asthma in strong susceptibility candidates such as the *IL4RA* gene, encoding the alpha chain of IL-4 and IL-13 receptors^{34, 57}.

We demonstrated functional differences for two asthma-associated SNPs. However, it remains unclear how the associated variants affect the S1PR1 activity *in vivo* and how such functional differences affect asthma susceptibility. To further explore the regulatory mechanisms of these promoter SNPs, we searched transcriptional factor binding sites potentially being interrupted by these variants and revealed EGFR and Egr2 sites for SNPs –1557 and –532, respectively. Epidermal growth factor (EGF) mediates airway smooth muscle proliferation and asthma remodeling⁵⁸ and S1P signaling plays an important role in EGF-mediated cell migration⁵⁹. Recent studies further showed that receptor transactivation between EGF receptor (EGFR) and S1PR1 receptors engages an additional mechanism involving HB-EGF shedding from the plasma membrane by metalloproteinases⁶⁰. *S1PR1* promoter SNP –1557T demonstrated less affinity to EGF stimulation (75% reduction) suggesting that S1PR1 transcription is regulated in response to EGF in endothelium. In contrast, the Egr2 transcriptional factor binding site acts as a transcriptional suppressor upon activation by several growth factors resulting in decreased gene expression of genes such as phosphatase 2A (PP2A)⁶¹. Platelet-derived growth factor (PDGF) and vascular endothelial cell growth factor (VEGF) are important factors in asthma and other inflammatory lung diseases^{5, 7, 10}, and significantly increase endogenous S1PR1 in endothelial cells^{62, 63}. VEGF treatment of vascular endothelium markedly up-regulates S1PR1 expression and enhances S1P-mediated signaling pathways leading to eNOS activation⁶⁴. Our data indicate that whereas *S1PR1* promoters with –532C and –532G alleles were responsive to PDGF and VEGF exposure, the –532G allele markedly and significantly influenced responses to PDGF and VEGF to a much greater degree than –532C, suggesting that variant –532G results in enhanced S1PR1 transcription in response to growth factors, potentially leading to an improved integrity of endothelial barrier or improved resolution of established vascular permeability. Since VEGF is well recognized to directly increase vascular permeability and asthma, and PDGF worsens asthma symptoms, the increased S1PR1 transcription could potentially counter effects of VEGF and PDGF on endothelial barrier dysfunction and

severity of asthma^{2, 65, 66}. These effects are consistent with the –532C allele conferring and increased risk for asthma.

In summary, while the genetic mechanisms underlying chronic inflammatory lung syndromes such as asthma remain incompletely understood, our studies demonstrated that protein expression of S1PR1, the endothelial dominant receptor for sphingosine-1-phosphate (S1P) and an essential participant in regulation of vascular integrity and angiogenesis, is decreased in asthmatics and that S1PR1 variants constitute risk factors for asthma. Thus, in agreement with the evidence supporting the benefits of asthma treatment with drugs targeting endothelial barrier enhancement, this data suggests that microvascular endothelium and S1PR1 may serve as potential targets for amelioration of asthma pathobiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AAs	African descent Americans
AIMs	ancestry informative markers
BAL	bronchoalveolar lavage
CI	confidence interval
CSGA	The Collaborative Study on the Genetics of Asthma
EAs	European descent Americans
EGF	epidermal growth factor
EGFR	Epidermal growth factor receptor
Egr2	Early growth response gene 2
FEV1	forced expiratory volume
HPAEC	Human pulmonary artery endothelial cells
HWE	Hardy-Weinberg equilibrium
MAF	Minor Allele Frequency
OR	odds ratio
PDGF	platelet-derived growth factor
S1P	sphingosine-1-phosphate
S1PR1	sphingosine-1-phosphate receptor 1
S1PR1^{-/-}	Deletion of the S1PR1 gene

SNPs	single nucleotide polymorphisms
TRANSFAC	transcription factor motif analysis
tSNPs	tagging SNPs
VEGF	vascular endothelial growth factor

REFERENCES

1. Naureckas ET, Ndukwu IM, Halayko AJ, Maxwell C, Hershenson MB, Solway J. Bronchoalveolar lavage fluid from asthmatic subjects is mitogenic for human airway smooth muscle. *Am J Respir Crit Care Med.* 1999; 160:2062–2066. [PubMed: 10588629]
2. Kanazawa H, Nomura S, Yoshikawa J. Role of microvascular permeability on physiologic differences in asthma and eosinophilic bronchitis. *Am J Respir Crit Care Med.* 2004; 169:1125–1130. [PubMed: 15044203]
3. Chetta A, Zanini A, Torre O, Olivieri D. Vascular remodelling and angiogenesis in asthma: morphological aspects and pharmacological modulation. *Inflamm Allergy Drug Targets.* 2007; 6:41–45. [PubMed: 17352687]
4. Simoes DC, Vassilakopoulos T, Toumpanakis D, Petrochilou K, Roussos C, Papapetropoulos A. Angiopoietin-1 protects against airway inflammation and hyperreactivity in asthma. *Am J Respir Crit Care Med.* 2008; 177:1314–1321. [PubMed: 18356565]
5. Lee CG, Link H, Baluk P, Homer RJ, Chapoval S, Bhandari V, et al. Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. *Nat Med.* 2004; 10:1095–1103. [PubMed: 15378055]
6. Asai K, Kanazawa H, Otani K, Shiraishi S, Hirata K, Yoshikawa J. Imbalance between vascular endothelial growth factor and endostatin levels in induced sputum from asthmatic subjects. *J Allergy Clin Immunol.* 2002; 110:571–575. [PubMed: 12373263]
7. Kanazawa H, Hirata K, Yoshikawa J. Involvement of vascular endothelial growth factor in exercise induced bronchoconstriction in asthmatic patients. *Thorax.* 2002; 57:885–888. [PubMed: 12324676]
8. Ohno I, Nitta Y, Yamauchi K, Hoshi H, Honma M, Woolley K, et al. Eosinophils as a potential source of platelet-derived growth factor B-chain (PDGF-B) in nasal polyposis and bronchial asthma. *Am J Respir Cell Mol Biol.* 1995; 13:639–647. [PubMed: 7576701]
9. Amishima M, Munakata M, Nasuhara Y, Sato A, Takahashi T, Homma Y, et al. Expression of epidermal growth factor and epidermal growth factor receptor immunoreactivity in the asthmatic human airway. *Am J Respir Crit Care Med.* 1998; 157:1907–1912. [PubMed: 9620926]
10. Simeone-Penney MC, Severgnini M, Roza L, Takahashi S, Cochran BH, Simon AR. PDGF-induced human airway smooth muscle cell proliferation requires STAT3 and the small GTPase Rac1. *Am J Physiol Lung Cell Mol Physiol.* 2008; 294:L698–L704. [PubMed: 18310224]
11. Panettieri RA, Tan EM, Ciocca V, Luttmann MA, Leonard TB, Hay DW. Effects of LTD4 on human airway smooth muscle cell proliferation, matrix expression, and contraction In vitro: differential sensitivity to cysteinyl leukotriene receptor antagonists. *Am J Respir Cell Mol Biol.* 1998; 19:453–461. [PubMed: 9730873]
12. English D, Welch Z, Kovala AT, Harvey K, Volpert OV, Brindley DN, et al. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. *Faseb J.* 2000; 14:2255–2265. [PubMed: 11053247]
13. English D, Kovala AT, Welch Z, Harvey KA, Siddiqui RA, Brindley DN, et al. Induction of endothelial cell chemotaxis by sphingosine 1-phosphate and stabilization of endothelial monolayer barrier function by lysophosphatidic acid, potential mediators of hematopoietic angiogenesis. *J Hematother Stem Cell Res.* 1999; 8:627–634. [PubMed: 10645770]
14. Garcia JG, Liu F, Verin AD, Birukova A, Dechert MA, Gerthoffer WT, et al. Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement. *J Clin Invest.* 2001; 108:689–701. [PubMed: 11544274]

15. Singleton PA, Dudek SM, Chiang ET, Garcia JG. Regulation of sphingosine 1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by S1P1 receptor, PI3 kinase, Tiam1/Rac1, and alpha-actinin. *FASEB J*. 2005; 19:1646–1656. [PubMed: 16195373]
16. Brinkmann V, Baumruker T. Pulmonary and vascular pharmacology of sphingosine 1-phosphate. *Curr Opin Pharmacol*. 2006; 6:244–250. [PubMed: 16563863]
17. Peng X, Hassoun PM, Sammani S, McVerry BJ, Burne MJ, Rabb H, et al. Protective effects of sphingosine 1-phosphate in murine endotoxin-induced inflammatory lung injury. *Am J Respir Crit Care Med*. 2004; 169:1245–1251. [PubMed: 15020292]
18. McVerry BJ, Peng X, Hassoun PM, Sammani S, Simon BA, Garcia JG. Sphingosine 1-phosphate reduces vascular leak in murine and canine models of acute lung injury. *Am J Respir Crit Care Med*. 2004; 170:987–993. [PubMed: 15282202]
19. Moreno-Vinasco L, Jacobson JR, Bonde P, Sammani S, Mirzapourzadeh T, Vigneswaran WT, et al. Attenuation of rodent lung ischemia-reperfusion injury by sphingosine 1-phosphate. *Journal of Organ Dysfunction*. 2008; 4:106–114.
20. Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, et al. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Invest*. 2000; 106:951–961. [PubMed: 11032855]
21. Allende ML, Yamashita T, Proia RL. G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation. *Blood*. 2003; 102:3665–3667. [PubMed: 12869509]
22. Sammani S, Moreno-Vinasco L, Mirzapourzadeh T, Singleton PA, Chiang ET, Evenoski CL, et al. Differential Effects of S1P Receptors on Airway and Vascular Barrier Function in the Murine Lung. *Am J Respir Cell Mol Biol*. 2009:0223OC.
23. Camerer E, Regard JB, Cornelissen I, Srinivasan Y, Duong DN, Palmer D, et al. Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. *J Clin Invest*. 2009; 119:1871–1879. [PubMed: 19603543]
24. Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymskaya VP, et al. Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J*. 2001; 15:1212–1214. [PubMed: 11344091]
25. Baumruker T, Prieschl EE. The role of sphingosine kinase in the signaling initiated at the high-affinity receptor for IgE (FcεRI) in mast cells. *Int Arch Allergy Immunol*. 2000; 122:85–90. [PubMed: 10878486]
26. Prieschl EE, Csonga R, Novotny V, Kikuchi GE, Baumruker T. The balance between sphingosine and sphingosine-1-phosphate is decisive for mast cell activation after FcεRI triggering. *J Exp Med*. 1999; 190:1–8. [PubMed: 10429665]
27. Roviezzo F, Di Lorenzo A, Bucci M, Brancalione V, Vellecco V, De Nardo M, et al. Sphingosine-1-phosphate/sphingosine kinase pathway is involved in mouse airway hyperresponsiveness. *Am J Respir Cell Mol Biol*. 2007; 36:757–762. [PubMed: 17322125]
28. Idzko M, Hammad H, van Nimwegen M, Kool M, Muller T, Soullie T, et al. Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. *J Clin Invest*. 2006; 116:2935–2944. [PubMed: 17080194]
29. Rivera J, Proia RL, Olivera A. The alliance of sphingosine-1-phosphate and its receptors in immunity. *Nat Rev Immunol*. 2008; 8:753–763. [PubMed: 18787560]
30. Brey EM, Lalani Z, Johnston C, Wong M, McIntire LV, Duke PJ, et al. Automated selection of DAB-labeled tissue for immunohistochemical quantification. *J Histochem Cytochem*. 2003; 51:575–584. [PubMed: 12704205]
31. Kelly DJ, Zhang Y, Connelly K, Cox AJ, Martin J, Krum H, et al. Tranilast attenuates diastolic dysfunction and structural injury in experimental diabetic cardiomyopathy. *Am J Physiol Heart Circ Physiol*. 2007; 293:H2860–H2869. [PubMed: 17720766]
32. Antonarakis SE. 10 years of Genomics, chromosome 21, and Down syndrome. *Genomics*. 1998; 51:1–16. [PubMed: 9693027]
33. A genome-wide search for asthma susceptibility loci in ethnically diverse populations. The Collaborative Study on the Genetics of Asthma (CSGA). *Nat Genet*. 1997; 15:389–392. [PubMed: 9090385]

34. Ober C, Leavitt SA, Tsalenko A, Howard TD, Hoki DM, Daniel R, et al. Variation in the interleukin 4-receptor alpha gene confers susceptibility to asthma and atopy in ethnically diverse populations. *Am J Hum Genet.* 2000; 66:517–526. [PubMed: 10677312]
35. Lester LA, Rich SS, Blumenthal MN, Togias A, Murphy S, Malveaux F, et al. Ethnic differences in asthma and associated phenotypes: collaborative study on the genetics of asthma. *J Allergy Clin Immunol.* 2001; 108:357–362. [PubMed: 11544453]
36. Pesola GR, Xu F, Ahsan H, Sternfels P, Meyer IH, Ford JG. Predicting asthma morbidity in Harlem emergency department patients. *Acad Emerg Med.* 2004; 11:944–950. [PubMed: 15347544]
37. Meyer IH, Sternfels P, Fagan JK, Copeland L, Ford JG. Characteristics and correlates of asthma knowledge among emergency department users in Harlem. *J Asthma.* 2001; 38:531–539. [PubMed: 11714075]
38. Flores C, Ma SF, Maresso K, Ober C, Garcia JG. A variant of the myosin light chain kinase gene is associated with severe asthma in African Americans. *Genet Epidemiol.* 2007; 31:296–305. [PubMed: 17266121]
39. Ma SF, Flores C, Wade MS, Dudek SM, Nicolae DL, Ober C, et al. A common cortactin gene variation confers differential susceptibility to severe asthma. *Genet Epidemiol.* 2008; 32:757–766. [PubMed: 18521921]
40. Amundadottir LT, Sulem P, Gudmundsson J, Helgason A, Baker A, Agnarsson BA, et al. A common variant associated with prostate cancer in European and African populations. *Nat Genet.* 2006; 38:652–658. [PubMed: 16682969]
41. Tian C, Hinds DA, Shigeta R, Adler SG, Lee A, Pahl MV, et al. A genome-wide single-nucleotide-polymorphism panel for Mexican American admixture mapping. *Am J Hum Genet.* 2007; 80:1014–1023. [PubMed: 17557415]
42. Tian C, Hinds DA, Shigeta R, Kittles R, Ballinger DG, Seldin MF. A genome-wide single-nucleotide-polymorphism panel with high ancestry information for African American admixture mapping. *Am J Hum Genet.* 2006; 79:640–649. [PubMed: 16960800]
43. Kosoy R, Nassir R, Tian C, White PA, Butler LM, Silva G, et al. Ancestry informative marker sets for determining continental origin and admixture proportions in common populations in America. *Hum Mutat.* 2009; 30:69–78. [PubMed: 18683858]
44. Falush D, Stephens M, Pritchard JK. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics.* 2003; 164:1567–1587. [PubMed: 12930761]
45. Parra EJ, Marcini A, Akey J, Martinson J, Batzer MA, Cooper R, et al. Estimating African American admixture proportions by use of population-specific alleles. *Am J Hum Genet.* 1998; 63:1839–1851. [PubMed: 9837836]
46. Pritchard JK, Stephens M, Rosenberg NA, Donnelly P. Association mapping in structured populations. *Am J Hum Genet.* 2000; 67:170–181. [PubMed: 10827107]
47. Weale ME, Depondt C, Macdonald SJ, Smith A, Lai PS, Shorvon SD, et al. Selection and evaluation of tagging SNPs in the neuronal-sodium-channel gene SCN1A: implications for linkage-disequilibrium gene mapping. *Am J Hum Genet.* 2003; 73:551–565. [PubMed: 12900796]
48. Ding K, Kullo IJ. Methods for the selection of tagging SNPs: a comparison of tagging efficiency and performance. *Eur J Hum Genet.* 2007; 15:228–236. [PubMed: 17164795]
49. Wigginton JE, Cutler DJ, Abecasis GR. A note on exact tests of Hardy-Weinberg equilibrium. *Am J Hum Genet.* 2005; 76:887–893. [PubMed: 15789306]
50. Storey JD, Tibshirani R. Statistical significance for genome-wide studies. *Proc Natl Acad Sci U S A.* 2003; 100:9440–9445. [PubMed: 12883005]
51. Singleton PA, Dudek SM, Ma SF, Garcia JG. Transactivation of sphingosine 1-phosphate receptors is essential for vascular barrier regulation. Novel role for hyaluronan and CD44 receptor family. *J Biol Chem.* 2006; 281:34381–34393. [PubMed: 16963454]
52. Dantas AP, Igarashi J, Michel T. Sphingosine 1-phosphate and control of vascular tone. *Am J Physiol Heart Circ Physiol.* 2003; 284:H2045–H2052. [PubMed: 12742827]

53. Huang YL, Lin HS, Chen SU, Lee H. Tyrosine sulphation of sphingosine 1-phosphate 1 (S1P1) is required for S1P-mediated cell migration in primary cultures of human umbilical vein endothelial cells. *J Biochem.* 2009; 146:815–820. [PubMed: 19692429]
54. Greene CS, Penrod NM, Williams SM, Moore JH. Failure to replicate a genetic association may provide important clues about genetic architecture. *PLoS One.* 2009; 4:e5639. [PubMed: 19503614]
55. Kurz T, Hoffjan S, Hayes MG, Schneider D, Nicolae R, Heinzmann A, et al. Fine mapping and positional candidate studies on chromosome 5p13 identify multiple asthma susceptibility loci. *J Allergy Clin Immunol.* 2006; 118:396–402. [PubMed: 16890764]
56. McCarthy MI, Abecasis GR, Cardon LR, Goldstein DB, Little J, Ioannidis JP, et al. Genome-wide association studies for complex traits: consensus, uncertainty and challenges. *Nat Rev Genet.* 2008; 9:356–369. [PubMed: 18398418]
57. Loza MJ, Chang BL. Association between Q551R IL4R genetic variants and atopic asthma risk demonstrated by meta-analysis. *J Allergy Clin Immunol.* 2007; 120:578–585. [PubMed: 17586032]
58. Krymskaya VP, Hoffman R, Eszterhas A, Kane S, Ciocca V, Panettieri RA Jr. EGF activates ErbB-2 and stimulates phosphatidylinositol 3-kinase in human airway smooth muscle cells. *Am J Physiol.* 1999; 276:L246–L255. [PubMed: 9950886]
59. Le Stunff H, Mikami A, Giussani P, Hobson JP, Jolly PS, Milstien S, et al. Role of sphingosine-1-phosphate phosphatase 1 in epidermal growth factor-induced chemotaxis. *J Biol Chem.* 2004; 279:34290–34297. [PubMed: 15180992]
60. Hofmann U, Burkard N, Vogt C, Thoma A, Frantz S, Ertl G, et al. Protective effects of sphingosine-1-phosphate receptor agonist treatment after myocardial ischaemia-reperfusion. *Cardiovasc Res.* 2009; 83:285–293. [PubMed: 19416991]
61. Yu L, Haverty PM, Mariani J, Wang Y, Shen HY, Schwarzschild MA, et al. Genetic and pharmacological inactivation of adenosine A2A receptor reveals an Egr-2-mediated transcriptional regulatory network in the mouse striatum. *Physiol Genomics.* 2005; 23:89–102. [PubMed: 16046619]
62. Ryu Y, Takuwa N, Sugimoto N, Sakurada S, Usui S, Okamoto H, et al. Sphingosine-1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. *Circ Res.* 2002; 90:325–332. [PubMed: 11861422]
63. Igarashi J, Miyoshi M, Hashimoto T, Kubota Y, Kosaka H. Hydrogen peroxide induces S1P1 receptors and sensitizes vascular endothelial cells to sphingosine 1-phosphate, a platelet-derived lipid mediator. *Am J Physiol Cell Physiol.* 2007; 292:C740–C748. [PubMed: 16943246]
64. Igarashi J, Erwin PA, Dantas AP, Chen H, Michel T. VEGF induces S1P1 receptors in endothelial cells: Implications for cross-talk between sphingolipid and growth factor receptors. *Proc Natl Acad Sci U S A.* 2003; 100:10664–10669. [PubMed: 12963813]
65. Hobson JP, Rosenfeldt HM, Barak LS, Olivera A, Poulton S, Caron MG, et al. Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. *Science.* 2001; 291:1800–1803. [PubMed: 11230698]
66. Yamashita N, Sekine K, Miyasaka T, Kawashima R, Nakajima Y, Nakano J, et al. Platelet-derived growth factor is involved in the augmentation of airway responsiveness through remodeling of airways in diesel exhaust particulate-treated mice. *J Allergy Clin Immunol.* 2001; 107:135–142. [PubMed: 11150003]

A. **Non-asthmatic lung****Asthmatic lung****B.** **Figure 1.**

Levels of S1PR1 protein expression in pulmonary vessels detected by immunohistochemistry staining. **(A)** Illustrative images of the frozen human lung sections from asthmatic and non-asthmatic individuals stained with anti-S1PR1 antibody. Arrows indicate S1PR1 protein staining in pulmonary arteries. **(B)** The fold change (y-axis) of S1PR1 protein level detected in non-asthmatics and asthmatics in this bar graph (* $p < 0.05$, vs. non-asthmatics).

Figure 2. Plots of association p -values of tested *SIPRI* tSNPs with asthma and severe asthma in (A) European Americans from Chicago, (B) African Americans from Chicago, and (C) African Americans from New York. Dashed line represents a p -value of 0.05. The *SIPRI* gene structure indicating the relative position of tested tSNPs for association is depicted in the bottom of the figure.

Figure 3. *In silico* analysis and promoter activity assay revealed

(A) A putative EGFR and Egr2 transcription factor binding site at positions -1557 (rs2038366) and -532 (rs59317557). (B) Effect of -1557 G/T SNP on *S1PR1* promoter activity under basal (no stimulation) and upon EGF (100 ng/ml) stimulations (* $p < 0.05$ vs. -1557G; # $p < 0.05$ vs. No EGF), (C) Effect of -532 C/G SNP on *S1PR1* promoter activity under basal (no stimulation) and upon PDGF (50 ng/ml) or VEGF (50 ng/ml) stimulation (* $p < 0.05$ vs. -532G; ** $p < 0.01$ vs. -532G; # $p < 0.05$ vs. No PDGF or VEGF).

Table 1

Summary of variation (tSNPs in bold) found by re-sequencing the *S/PR1* gene and 2kb flanking sequences in European (EAs) and African Americans (AAs).

SNP#	Position ^a	Location (effect) ^b	rs #	Flanking (5')	Flanking (3')	MAF ^c (AA)	MAF ^c (EA)	Val ^d
1	101473084	-195G>A ^d	-	GAAAAATGAGT	GTTTATAAAG	0.00	0.04	-
2	101473486	-1557G>T ^d	rs2038366	-	-	0.14	0.23	†
3	101473532	-1511G>T ^d	rs17123892	-	-	0.05	0.00	‡
4	101473700	-1343A>C ^d	-	CTGTTCGATGA	TGCTTTAAAA	0.04	0.04	-
5	101474020	-1023T>C ^d	rs12407294	-	-	0.14	0.23	‡
6	101474069	-974T>C ^d	rs33967958	-	-	0.39	0.42	†
7	101474427	-616C>T ^d	-	ACAGGAACCT	CCGGTTTCATT	0.11	0.00	-
8	101474511	-532C>G ^d	rs59317557	-	-	0.39	0.42	†
9	101474558	-485G>C ^d	-	TCGCGCGAAA	TGGCTCGGGG	0.04	0.00	-
10	101475134	c.-273G>C	rs4987248	-	-	0.04	0.08	‡
11	101475136	c.-271delG	rs3835397	-	-	0.25	0.38	†
12	101475176	c.-231A>G	rs3765684	-	-	0.04	0.08	‡
13	101475297	c.-164+54G>A	-	GGTTGTGGTA	GGCTTAGGTG	0.04	0.00	-
14	101475412	c.-164+169G>A	-	AGCCCATCTC	ATCAGATTGT	0.04	0.00	-
15	101475413	c.-164+170A>G	rs3753194	-	-	0.04	0.08	‡
16	101475837	c.-164+594T>C	rs1411017	-	-	0.32	0.19	‡
17	101475939	c.-164+696G>C	-	TTTTTGCAATC	AAATTAGAAA	0.04	0.00	-
18	101476041	c.-164+798T>C	rs17100952	-	-	0.07	0.00	‡
19	101476067	c.-164+824T>C	-	ACGAAACATTC	GGGATTGCT	0.04	0.00	-
20	101476144	c.-163-822delG	-	GGGTGGGGG	TGGGGTGGGG	0.07	0.00	-
21	101476159	c.-163-807G>T	rs56236417	-	-	0.11	0.00	†
22	101476236	c.-163-730A>T	rs61780687	-	-	0.14	0.23	†
23	101476497	c.-163-469G>A	-	TCCGAGGAGA	ACGTGGGCTG	0.04	0.00	-
24	101477081	c.-48G>A	-	TCTAGCGTTC	TCTGGAGTAG	0.04	0.00	-
25	101477084	c.-45T>C	rs3737578	-	-	0.04	0.15	‡

SNP#	Position ^a	Location (effect) ^b	rs #	Flanking (5')	Flanking (3')	MAF ^c (AA)	MAF ^c (EA)	Val ^d
26	101477120	c.-9G>T	rs3737577	-	-	0.07	0.27	†
27	101477160	c.32C>A (p.Ala11Asp)	-	CTGGTCAAGG	CCACCGCAGC	0.04	0.00	-
28	101477165	c.37C>G (p.Arg13Gly)	rs41287280	-	-	0.00	0.04	†
29	101478834	c.*557T>C	-	CGATCATCTA	AGCAAAATAGG	0.00	0.08	-
30	101478994	c.*717C>A	rs55872049	-	-	0.07	0.00	†
31	101479030	c.*753C>T	-	CCATTGAAGC	GAAATCTGCA	0.04	0.00	-
32	101479328	c.*1051T>C	-	AGTCATTTTA	TGGATTTTTC	0.04	0.00	-
33	101479565	c.*1288dupT	-	TTGTTTTTTTT	AAAAAGAATA	0.07	0.00	-
34	101479714	*53T>C ^f	rs12090529	-	-	0.25	0.00	†
35	101479716	*55T>A ^f	-	TACTGGATTG	GTAGCTCTTT	0.04	0.00	-
36	101479862	*201A>G ^f	-	TTAACACTGA	GAGTAGTAAA	0.04	0.00	-
37	101481168	*1507delA ^f	rs35079261	-	-	0.14	0.08	†
38	101481340	*1679T>C ^f	rs12409786	-	-	0.04	0.15	†
39	101481435	*1774T>A ^f	rs1358733	-	-	0.18	0.23	†

^aPosition in chromosome 1 according to NCBI build 36

^bReference sequence is NM_001400.3

^cMinor allele frequency

^dValidation status: frequency available,

† unknown or by 2-hit as in dbSNP build 129, †

^eDistance from first nucleotide of NM_001400.3

^fDistance from last nucleotide of NM_001400.3

Table 2

Minor allele frequency for the *S/PR* tSNPs genotyped.

tSNP	Chicago samples						New York samples		
	European Americans			African Americans			African Americans		
	Controls	Asthma	Severe asthma	Controls	Asthma	Severe asthma	Controls	Asthma	Severe asthma
rs2038366	0.32	0.42	0.44	0.26	0.30	0.30	0.28	0.29	0.18
rs17123892	0.00	0.00	0.00	0.06	0.07	0.07	0.08	0.06	0.03
rs59317557	0.39	0.40	0.35	0.43	0.46	0.44	0.45	0.39	0.25
rs3753194	0.08	0.06	0.12	0.07	0.03	0.03	0.04	0.06	0.05
rs1411017	0.05	0.04	0.04	0.34	0.35	0.32	0.35	0.32	0.33
rs61780687	0.35	0.40	0.48	0.10	0.09	0.09	0.08	0.06	0.05
rs3737577	0.34	0.30	0.37	0.06	0.06	0.07	0.03	0.05	0.11
SNP 29	0.01	0.01	0.02	<0.01	<0.01	0.02	0.00	0.00	0.00
rs12090529	0.04	0.04	0.04	0.28	0.25	0.27	0.28	0.33	0.25
rs1358733	0.18	0.20	0.24	0.25	0.20	0.20	0.27	0.23	0.23